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FOR

HUMAN BRAIN ENDOTHELIAL CELLS AND GROWTH MEDIUM AND METHOD FOR EXPANSION OF PRIMITIVE CD34+CD38- BONE MARROW STEM CELLS

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HUMAN BRAIN ENDOTHELIAL CELLS AND GROWTH MEDIUM AND METHOD FOR EXPANSION OF PRIMITIVE CD34+CD38- BONE MARROW STEM CELLS

Background of the Invention

Related Application

Benefit of U.S. Provisional Application 60/112,042 filed 04 December 1998, which is incorporated herein by reference, is claimed.

Field of the Invention

This invention relates to a growth medium derived from human brain endothelial cells (HUBEC) and the methods of utilizing said growth medium to expand bone marrow stem cells.

Description Of The Prior Art

The development of an ex-vivo system which supports the proliferation and expansion of the most primitive hematopoietic stem cells (HSC) would have direct application to the fields of gene therapy and stem cell transplantation. Identification and characterization of the optimal culture conditions for the expansion of long-term repopulating cells is a requirement for gene therapy protocols and other stem cell-based therapies.

Various cytokine combinations and liquid culture methods have been shown to support the proliferation of $CD34^+$ HPC in vitro, but the most primitive $CD34^+CD38^-$ cells are frequently lost due to differentiation and cell death [1-6]. In contrast, other investigations have demonstrated that when human HPC are co-cultured in contact with autologous, allogeneic, and xenogeneic bone marrow stroma, a small percentage of long term culture initiating cells (LTC-IC) can be maintained over several weeks [7-9].

Similarly, others have reported the expansion and differentiation of LTC-IC and CFC in stroma-free liquid suspension cultures using exogenous cytokines plus conditioned medium from bone marrow stromal cultures [10-12]. Most recently, it was reported that human cord blood $CD34^+$ cells could be maintained in stroma-free liquid cultures in the presence of flt-3 ligand, megakaryocyte growth and development factor (MGDF), SCF, and IL-6 for up to 10 weeks without losing their ability to repopulate NOD/SCID mice [13].

Vascular endothelium, reticuloendothelial elements, and hematopoietic cells of all types have been postulated to arise from hemangioblasts, a primitive embryonic cell of mesodermal origin [14,15]. During the earliest stages (day 7-8 postcoitum) of mammalian embryonic hematopoiesis, primitive hematopoietic stem cells are found encased in blood islands which derive from aggregates of mesodermal cells which have colonized the embryonic yolk sac [16]. Bone marrow, umbilical vein, and murine yolk sac endothelial cell lines have been shown to elaborate a number of growth factors that regulate early hematopoiesis [17-20]. In addition, the long term proliferation and differentiation of myeloid, erythroid, and megakaryocytic progenitor cells has been demonstrated in vitro using microvascular endothelial cells derived from adult bone marrow and embryonic yolk sac [18,19]. However, the fate of the most primitive $CD34^+CD38^-$ progenitor cells following co-culture with endothelial cell monolayers has not been well demonstrated. Previously, we reported that a primary porcine microvascular endothelial cell line (PMVEC) supports a rapid and robust expansion of human hematopoietic cells exhibiting the primitive $CD34^+CD38^-$ phenotype [21,22]. Unlike other reported co-culture systems, we have demonstrated that $CD34^+CD38^-$ cells

expanded on brain endothelium retain the ability to successfully engraft in vivo in both a SCID-Hu bone model [23] and in lethally irradiated baboons [24].

Human brain vascular endothelial cells are similar to other sources of endothelial cells in that they develop cobblestone morphology in-vitro [25], and they express cell adhesion molecules (selectins, integrins) which mediate the "rolling", adherence, and trafficking of leukocytes [26,27]. Based upon our observations of the hematopoietic capacity of PMVEC and recognizing the limitations of applying a porcine endothelial cell line in human clinical studies, we isolated primary human brain endothelial cells (HUBEC) and evaluated their capacity to support the ex-vivo expansion of human $CD34^+CD38^-$ cells. Our results indicate that human brain endothelial cells support a unique expansion and apparent self-renewal of the most primitive $CD34^+CD38^-$ HPC at a level comparable to our observations with porcine endothelial cells. Further investigations evaluating the in vivo repopulating potential of HUBEC-expanded HPC will be important in implementing future gene therapy, cord blood expansion, and stem cell transplant protocols.

SUMMARY OF THE INVENTION

Accordingly, an object of this invention is a growth medium based on human brain endothelial cells (HUBEC).

Another object of the invention is the growth factor contained within the medium that is elaborated by the HUBEC and promotes the expansion of primitive CD34+CD38- bone marrow stem cells.

A further object of this invention is a method for expanding the population of primitive CD34+ CD38- bone marrow stem cells.

Yet another object of this invention is the treated, concentrated product of the growth medium containing the growth factor.

An additional object of the invention is a growth medium that can be used for GMP production of expanded cells.

These and additional objects of the invention are accomplished by human brain endothelial cells (HUBEC) that can serve as a uniquely supportive hematopoietic microenvironment.

BRIEF DESCRIPTION OF THE DRAWINGS

A more complete appreciation of the invention will be readily obtained by reference to the following Description of the Preferred Embodiments and the accompanying drawings in which like numerals in different figures represent the same structures or elements. The representations in each of the figures are diagrammatic and no attempt is made to indicate actual scales or precise ratios. Proportional relationships are shown as approximations.

Figure 1. Phenotype of HUBEC in primary culture. A. typical cobblestone morphology of HUBEC (passage 10) from a confluent (40 X magnification). B. Von Willebrand expression by cultured HUBEC (passage 10) was analyzed by flow cytometeric analysis. Isotype-matched control Ab is indicated by a heavy solid line while FITC-conjugated anti-human Von Willebrand staining is depicted by the dotted line.

Figure 2. Morphology of a typical adherent colony of hematopoietic cells following 7 days of co-culture of human bone marrow CD34+ cells on HUBEC

monolayers treated with Granulocyte monocyte colony stimulating factor (GMCSF) + Interleukin-3 (IL-3) + Interleukin-6 (IL-6) + Stem cell factor (SCF)+ fetal liver tyrosine kinase-3 ligand (flt-3 ligand).

A) Dispersed colony of hematopoietic cells adherent to HUBEC monolayers after vigorous washing (40 x).

B) B) Adherent colony of hematopoietic cells on HUBEC monolayer stained with Wrights' Geimsa stain (100 x).

Figure 3. Flow cytometric analysis of expanded CD34+ bone marrow cells following HUBEC co-culture vs. stroma-free liquid culture vs. Human non-brain endothelial cell co-culture. Purified human CD34+ cells were seeded on HUBEC monolayers or in stroma-free liquid culture or in co-culture with non-brain endothelial cell monolayers in the presence of GMCSF + IL-3 + IL-6 + SCF + flt-3 ligand and cultured for 7 days. The phenotype of purified bone marrow CD34+ cells at day 0 (input) is shown in (A). After 7 days, non-adherent hematopoietic cells were harvested from the HUBEC co-cultures (B), the liquid suspension cultures (C), and the non-brain endothelial cell co-cultures (D), and stained with FITC-conjugated CD34 MoAb and PE-conjugated CD38 MoAb and analyzed by flow cytometry. Log fluorescence distribution of CD34 expression is shown along the X-axis and CD38 expression along the Y-axis. Each result is shown with its isotype control.

Figure 4. Morphology of the CD34+CD38- cells following 7 days of HUBEC co-culture. Following 7 days of co-culture of human CD34+ cells on HUBEC monolayers treated with GMCSF + IL-3 + IL-6 + SCF + flt-3 ligand, the non-adherent cells were harvested and stained with FITC-conjugated CD34 and PE-conjugated CD38.

Representative CD34+CD38- cells collected by Fluorescence activated cell sorting (FACS) and stained with Wrights' Geimsa are shown at 100x.

Figure 5. Cell cycle status of bone marrow CD34+CD38- cells at day 0 and following 7 days of HUBEC co-culture. Bone marrow CD34+ cells were stained with CD34APC, CD38PE, Ki67FITC, and 7AAD to assess for cell cycle status.

In (A), bone marrow CD34+CD38- cells are shown based upon their staining for Ki67FITC and 7AAD.

In (B), CD34+CD38- cells are shown using the same stains.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Bone marrow $CD34^{+}CD38^{-}$ cells are highly enriched for pluripotent progenitor cells which account for long term repopulation in vivo [31-33], but attempts at expanding $CD34^{+}CD38^{-}$ cells in-vitro for therapeutic use have had very limited success due to the differentiation and cell death which frequently occurs when these primitive cells are exposed to cytokines [3,6,34]. An ex-vivo co-culture system which has the capacity to expand the population of long term repopulating cells while maintaining their $CD34^{+}CD38^{-}$ phenotype would have immediate clinical applications in gene therapy, cord blood expansion, and stem cell transplantation protocols.

In this study, we demonstrate that primitive hematopoietic progenitor/stem cells actively proliferate and expand in direct association with preformed HUBEC monolayers, which is consistent with our previous observations using a porcine endothelial cell line [21]. Unlike liquid suspension cultures and non-CNS derived endothelial cell cultures, co-culture with HUBEC is essential for expansion of the primitive $CD34^{+}CD38^{-}$ subset

(440-fold at day 7) while maintaining their primitive phenotype and immature undifferentiated blast cell morphology. In addition, $CD34^+$ cell proliferation in HUBEC co-culture appears to be the greatest in the $CD34^+CD38^-$ cell population. While the addition of exogenous growth factors including GM-CSF, IL-3, IL-6, SCF and flt3-ligand are important for $CD34^+$ cell activation and expansion, additional as-yet unidentified endothelial cell factors most likely play a critical role in the $CD34^+CD38^-$ cell "self-renewal" processes [35]. In contrast, results from stroma-free and non-brain endothelial cell cultures demonstrate that cultured $CD34^+CD38^-$ fail to proliferate significantly, differentiate quickly, and overall CFC cell expansion is limited and short lived. These results suggest that brain-derived endothelial cells provide a unique microenvironment which promotes the cell division and apparent self-renewal of the primitive $CD34^+CD38^-$ population.

We confirm that the majority of steady state bone marrow $CD34^+CD38^-$ cells are quiescent and reside primarily in G_0 of the cell cycle [22,36,37]. The lack of cell cycling induction within the most primitive $CD34^+CD38^-$ population has been identified as a major impediment to the successful transduction of these cells with retroviral based gene vectors [36,38]. We have previously determined that $CD34^+CD38^-$ cells are easily recruited into cell cycle when cultured on porcine brain endothelial cells (PMVEC) [22]. In the current study, we found that primary HUBEC cultures from numerous donors in combination with exogenous cytokines induced the majority (>70%) of the previously quiescent $CD34^+CD38^-$ population to enter G_1 or G_2/S/M phase of cell cycle after 7 days. Although the mechanism of rapid expansion of $CD34^+CD38^-$ cells is unclear, HUBEC may provide the microenvironment necessary in combination with exogenous cytokines

to induce rapid cycling and preserve the "stemness" of very primitive HPC (<2% of the total $CD34^+$ cells used to initiate cultures) and may also prevent apoptotic cell death. In contrast, we have found that this high level of cell division in $CD34^+CD38^-$ cells is not achievable in stroma-free liquid and human non-brain endothelial cocultures supplemented with GMCSF + IL-3 + IL-6 + SCF + flt-3 ligand. Unlike other stromal based culture systems [10,11], we do not observe inhibitory effects of endothelial cell contact on $CD34^+CD38^-$ expansion. In fact, in the HUBEC system, cell-to-cell contact promotes maximal expansion of the $CD34^+CD38^-$ cell population which is dependent upon the addition of a combination of exogenous growth factors and appears to override any type of direct endothelial cell inhibitory effects. These findings are consistent with our previously reported observations that $CD34^+CD38^-$ cell expansion is optimal when $CD34^+$ cells are cultured directly in contact with PMVEC monolayers rather than when cultured separately from the endothelial feeder cells using transwell inserts [21]. Since HUBEC provide a microenvironment which supports a high level of cell cycling and expands the primitive $CD34^+CD38^-$ population, this culture system may also promote higher efficiencies of gene transfer into transplantable cells using standard retroviral vectors.

Bone marrow $CD34^+CD38^-$ cells contain long term culture initiating cells (LTC-IC) which give rise to CFC over 6 weeks when cultured with stromal feeder layers [29,39]. Our clonogenic data in this study is consistent with that reported by others demonstrating that steady state $CD34^+CD38^-$ cells do not give rise to significant numbers of CFC when cultured directly in 14 day methylcellulose cultures plus cytokines [29,39]. For that reason, $CD34^+CD38^-$ cells typically have been

characterized as having limited CFC activity. It has previously been reported that 10 day serum-free liquid cultures of human $CD34^+CD38^-$ cells with optimal cytokine combinations including flt-3 ligand, SCF, and IL-3 promoted a 30-fold increase in LTC-IC production by the expanded population [30]. Although the authors did not address the phenotype of the expanded population in that study, it is likely that the majority of the input $CD34^+CD38^-$ cells exposed to cytokines for 10 days underwent significant differentiation and lineage commitment. In contrast, we demonstrate in this study that co-culture of human $CD34^+CD38^-$ cells with preformed HUBEC monolayers plus cytokines supports rapid cycling and ex vivo expansion of phenotypically primitive HPC of the $CD34^+CD38^-$ subset. Since the HUBEC co-culture supports this pronounced increase in $CD34^+CD38^-$ cells, we were easily able to collect and study this rare subset of long term repopulating cells to interrogate their biology after 7 days of co-culture. Unlike $CD34^+CD38^-$ cells in the steady state, $CD34^+CD38^-$ cells expanded on HUBEC monolayers directly give rise to hundreds of colonies of myeloid, erythroid, and mixed lineages in methylcellulose at a cloning efficiency of 24%. This suggests a period of pre-incubation in HUBEC co-culture plus cytokines can stimulate early HPC (stromal cell responsive progenitor cells) which would normally be cytokine unresponsive in a stroma-free microenvironment. In previous studies, we have shown that HSC expanded in PMVEC coculture are capable of competitive myeloid and lymphoid marrow repopulating when implanted into SCID-hu-bone and transplanted into lethally irradiated baboons [23,24]. Together these findings demonstrate the requirement for direct stem cell-stromal cell interaction in order to optimize HPC survival, expansion, and maintenance of HPC function under ex-vivo culture conditions and to preserve graft

quality. Moreover, the ability to determine stroma cell dependent CFC frequencies in a short time interval makes the HUBEC culture system an attractive alternative to other long-term in vitro quantification methodologies. Likewise, the ability to activate and significantly expand $CD34^+CD38^-$ progenitor cell pool has potential ramifications in clinical stem cell expansion studies.

Recently, bone marrow microvascular and human umbilical vein endothelial lines have been used to support the short-term growth and proliferation of human $CD34^+$ progenitor cells [18,20]. However, the outcome of the primitive $CD34^+CD38^-$ subpopulation has not been detailed in these co-culture systems. More recently, a stromal cell line derived from murine fetal liver, AFT024, has been shown to support the maintenance of a small percentage of $CD34^+CD38^-$ cells over 3-10 days of co-culture [40]. The authors hypothesized in this study that the AFT024 cell line may have maintained extended long term culture initiating cells (ELTC-ICs) by inhibiting cell cycling and differentiation of these $CD34^+CD38^-$ cells [40]. In contrast to these observations, HUBEC co-culture induces a high level of cell cycling in the quiescent $CD34^+CD38^-$ subset and the absolute percentage of $CD34^+CD38^-$ cells is not only maintained, but increases ~ 440-fold (0.3% at day 0 to 10.5%) at day 7. These data suggest that human brain endothelial cells may provide other hematopoietic signal(s) such as soluble growth factors, membrane-bound growth factors, extracellular matrix proteins, or cellular adhesion molecules, which are unique from fetal liver, bone marrow, or umbilical vein endothelial cell lines.

Since human brain endothelial cells support the apparent self renewal and expansion of primitive HPC whereas non-brain endothelial cells from the same donor do not, we

speculate that the biology of brain endothelial cells may be similar to embryonic and extra-embryonic endothelial cells which are critically involved in the generation of hematopoietic stem cells during embryogenesis [14-16]. Recently, it was reported that a murine aorto-gonad-mesonephros (AGM) region derived endothelial cell line (DAS 104-4) was capable of maintaining a small fraction of murine $CD34^+$ $Sca\text{-}1^+$ $c\text{-}kit^+$ lin^- cells over 7 days of co-culture and these hematopoietic cells retained their in vivo reconstituting capacity [41]. Based upon their findings, the authors hypothesized that the DAS 104-4 AGM-derived cell line was able to support the self renewal of a small percentage of hematopoietic stem cells [41]. Based upon their similar capacities to maintain primitive hematopoietic progenitor cells ex vivo, it is plausible that human brain endothelial cells may possess similar hematopoietic properties to AGM-derived endothelial cells. In addition, the profound induction of cell cycling and expansion of the $CD34^+CD38^-$ subpopulation observed on HUBEC monolayers suggests that brain endothelial cells may be providing novel hematopoietic signals as well.

In comparison to other ex-vivo cultures systems, we believe that the human brain endothelial cell (HUBEC) culture system has several major advantages which will prove useful in future clinical stem cell expansion and gene therapy studies. First, rapid amplification and collection of very large numbers cycling $CD34^+CD38^-$ cells can occur within 1 week of culture. Second, expansion of hematopoietic progenitor cells requires only preformed endothelial monolayers, which are easy to establish and maintain, plus a defined combination of commercially available cytokines. Third, we have shown that expansion of $CD34^+CD38^-$ cells requires only human brain endothelial cells (single cell type) whose hematopoietic biology should be more easily dissected compared to

heterogeneous stromal cell systems. Fourth, we have previously shown that HPCs expanded on porcine brain endothelial cell monolayers retain both in vivo myeloid and lymphoid repopulating potential with no apparent engraftment defects [23,24]. Results from ongoing SCID-Hu and primate bone marrow transplantation studies utilizing primitive $CD34^{+}CD38^{-}$ cells expanded on HUBEC monolayers will be important in evaluating this system for future therapeutic applications.

Definitions

1. Human CD34+ hematopoietic stem cells (HSC): CD34+ cells isolated from various hematopoietic tissues (not limited to peripheral blood, bone marrow, cord blood, spleen, and liver) that are capable of full and permanent lymphoid, erythroid and myeloid reconstitution following transplantation into a lethally irradiated recipient. These cells are a subset of the CD34+CD38- HPC population.
2. HPC: Hematopoietic progenitor cells
3. CD34+ CD38+ hematopoietic progenitor cells: Committed/differentiated CD34+ hematopoietic progenitor cells that express the lineage commitment surface marker CD38 and are functionally described as having only short-term hematopoietic reconstitution in vivo.
4. CD34+CD38- hematopoietic progenitor cells: The undifferentiated subset of CD34+ HPC cells that lacks CD38 expression and contains hematopoietic stem cells (HSC) which are functionally capable of long-term hematopoietic reconstitution. The HSC are located in the population of cells
5. SCF: Stem cell factor
6. IL-6: Interleukin-6
7. MGDF: Megakaryocyte growth and developmental factor
8. Long-term culture-initiating cells (LTC-IC): HSC that are defined by their potential to grow, proliferate and be maintained in stroma based cultures systems in the absence of exogenous cytokines over 5-7 weeks of culture.
9. Colony-forming cells (CFC): Committed progenitor cells (CD34+CD38+) that give rise to assayable in vitro colonies of either the myeloid, erythroid, or lymphoid lineages following 14 days of culture.
10. HUBEC: Human brain derived endothelial cells
11. GM-CSF: granulocyte macrophage colony-stimulating factor.
12. IL-3: Interleukin-3
13. MoAb: Monoclonal antibody
14. FACS: Fluorescent activated cell sorting
15. PE-CD38: Phycoerythrin conjugated anti-CD38 antibody
16. FITC-CD34: Fluorescein Isothiocyanate anti-CD34 antibody

17. SID: Surface intracellular DNA analysis
18. 7-AAD: 7-aminoactinomycin
19. FCS: Fetal bovine serum
20. CD34+Sca-1+ c-kit+ lin- cells: Primitive murine hematopoietic stem cells that have full and long-term hematopoietic reconstitution potential in vivo.
21. Flt3 (Rosnet et al. Oncogene, 6, 1641-1650, 1991) and flk-2 (Matthews et al., Cell, 65, 1143-1152, 1991) are variant forms of a TKR that is related to the c-fms and c-kit receptors. The flk-2 gene product is expressed on hematopoietic and progenitor cells, while the flt3 gene product has a more general tissue distribution. The flt3 and flk-2 receptor proteins are similar in amino acid sequence and vary at two amino acid residues in the extracellular domain and diverge in a 31 amino acid segment located near theC-termini (Lyman et al., Oncogene, 8, 815-822, 1993).
22. Flt3-ligand ("flt3-L") has been found to regulate the growth and differentiation of progenitor and stem cells and is likely to possess clinical utility in treating hematopoietic disorders, in particular, aplastic anemia and myelodysplastic syndromes. Additionally, flt3-L will be useful in allogeneic, syngeneic or autologous bone marrow transplants in patients undergoing cytoreductive therapies, as well as cell expansion. Flt3-L will also be useful in gene therapy and progenitor and stem cell mobilization systems.

EXAMPLES

Isolation and Culture of Primary Human Brain Endothelial Cells (HUBEC)

Short segments (< 10 cm) of blood vessels contained within the central nervous system (segments of the anterior cerebral artery and vertebro-basilar artery branching from the Circle of Willis) and segments of vessels from outside the CNS (internal iliac artery and renal artery) were obtained from autopsy specimens less than 12 hours post-mortem after informed consent was obtained. Blood vessel segments were placed in 4°C complete endothelial cell culture medium consisting of M199 (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT), 100 mcg/mL L-glutamine, 50 mcg/mL heparin, 30 mcg/mL endothelial cell growth factor supplement (Sigma, St. Louis, MO) and 100 mcg/mL penicillin/streptomycin solution.

Within 6 hours of primary dissection from the brain, blood vessels were gently washed twice with PBS (Ca2+, Mg2+ free) and transferred to gelatin-coated *(need size)* tissue culture dishes containing 2 mL of complete endothelial cell growth media. Using a sterile #10 scalpel blade, 1mm cross sectional cuts were made along the length of the vessels. Larger vessels were first cut longitudinally with three incisions, to open and flatten the vessel, and then inverted to orient the vessel lumen towards the surface of the tissue culture dish. Immediately following the dissection an additional 2 mL of complete endothelial cell media was added to each dish. Cultures were placed in a humidified 37°C, 5% CO_2 atmosphere.

Distinct macroscopic cobblestone HUBEC colonies were evident between days 7 - 14 of culture. Following the establishment of confluent monolayers (~30 days), spent culture medium was collected and endothelial cell monolayers were washed vigorously with PBS (Ca++, Mg++ Free), trypsinized (0.25 mg trypsin/mL, 5 mmol/L EDTA, 37o C, 10 minutes; GIBCO) and subcultured at a ratio of 1:5 into gelatin-coated 75 cm2 flasks (Costar, Cambridge, MA) containing 20 mL of complete endothelial cell culture medium. HUBEC monolayers were fed weekly with complete medium and several passages of the primary cells were established and banked.

Characteristics of Human Brain Endothelial Cells

HUBEC from passages 1-10 appeared morphologically identical with no observable differences in the rate of growth noted. Cultures developed the typical uniform endothelial cell monolayer cobblestone morphology when 80-100% confluent (Figure 1A). Cells at passages 5-10 were harvested using 5 mM EDTA and stained with a monoclonal antibody against human Von Willebrand Factor, and then analyzed by flow

cytometry. As shown in Figure 1B, Von Willebrand Factor is highly expressed on HUBEC. HUBEC do not express either the CD34 or CD38 antigen at significant levels (<5%, data not shown).

Expansion of Bone Marrow CD34+ Cells on HUBEC Monolayers

Human $CD34^+$ cells were isolated from normal human cadaveric bone marrow as previously described [21] with >96 % purity. The effects of HUBEC co-culture on $CD34^+$ cell proliferation and CFC generation were initially compared with stroma-free liquid suspension cultures and co-cultures utilizing human non-brain endothelial cells isolated from the same cadaveric donors. All cultures were treated identically with a combination of five stimulatory cytokines (GM-CSF + IL-3 + IL-6 + SCF + flt-3 ligand) previously shown to support optimal $CD34^+$ cell proliferation [21]. After 7 days of co-culture, large macroscopic colonies (>2000 cells) developed on HUBEC in which the majority of the cells could be dispersed and collected by gently washing of the HUBEC monolayers with culture medium. Remaining cells (<10 %) appeared to be tightly adherent and embedded within the HUBEC monolayer resembling "cobblestone-like hematopoietic foci" (Figure 2A & 2B). In the absence of exogenously supplied growth factors very little cell growth was observed. At day 7 of HUBEC coculture, the mean number of total nonadherent and $CD34^+$ cells increased 13.4- and 6.4-fold, respectively, with a 453-fold increase in the number of $CD34^+CD38^-$ cells (Table 1). Forty-eight percent of the harvested nonadherent cells following 7 days of HUBEC coculture expressed the CD34 antigen. The $CD34^+CD38^-$ subpopulation, defined as $CD34^+$ cells that expressed CD38PE fluorescence at least one half less than the PE-isotype control, increased from a mean of 0.3 % of the population at day 0 to 10.5 % of the total

nonadherent cell population at day 7 and constituted 21% of the day 7 expanded $CD34^+$ cell pool (Table 1). Figures 3A and 3B show a representative phenotype of bone marrow $CD34^+$ cells at day 0 (3A) and after 7 days of HUBEC co-culture (3B). As shown in Figure 4, $CD34^+CD38^-$ cells isolated by cell sorting from day 7 HUBEC co-cultures are primarily agranular blasts with a high nuclear to cytoplasmic ratio, a fine chromatin pattern, and prominent nucleoli.

We also compared the capacity of the HUBEC coculture system to expand $CD34^+CD38^-$ cells and multilineage CFC with stroma-free liquid suspension cultures and with non-brain endothelial cell cocultures using the identical combination of exogenous cytokines over 7-14 days of culture. Maximal nonadherent (233-fold) and total $CD34^+$ cell expansion (21-fold) was detected following 14 days of culture using the HUBEC coculture system (Table 2), with a 1690-fold increase in the absolute number of $CD34^+CD38^-$ cells. Additionally, CFU-GM, CFU-Mix and BFU-E CFC progenitors increased 558-, 129-, and 180-fold respectively (Table 3). In comparison, overall cell and CFC yields were significantly lower in stroma-free liquid suspension and in non-brain endothelial cell co-cultures (Tables 2 and 3). Total $CD34^+$ cell numbers were maintained or moderately increased ($\leq$7-fold) over 14 days under these culture conditions with little or no amplification of the $CD34^+CD38^-$ cell population detected following 7 days of ex vivo culture. Representative day 7 phenotypes of hematopoietic cells expanded in liquid suspension cultures and non-brain endothelial cell cocultures are shown in Figures 3C and 3D.

TABLE 1. Ex-vivo Expansion of Human Bone Marrow $CD34^+$ and $CD34^+CD38^-$ Cells in Cytokine-Treated HUBEC Cultures

	No. of Cells Procured x 10^5			
Culture Conditions	**Cell yield x 10^5**	**$CD34^+$**	**$CD34^+CD38^+$**	**$CD34^+CD38^-$**
Input $CD34^+$ Cells	5.0	5.0 (100)	4.85 (99.7)	0.015 (0.3)
HUBEC Co-culture (day 7)	67 ± 17.1 (100)	32 ± 7.0 (79)	25.3 ± 7.4 (21)	6.8 ± 3.9

$CD34^+$ BM cells (5 x 10^5) were plated per culture treatment. Nonadherent cells were procured on day 7 of culture. Cells of each culture were stained for phenotypic analysis with FITC-conjugated CD34 (HPCA-2) plus PE-conjugated CD38). Stained cells were analyzed using two-color flow cytometry. The number of each immunophenotype was corrected to reflect the total number of cells procured/culture. Each point represents the mean number of positive cells from five different experiments. Numbers in parentheses indicate the relative frequency of a given phenotype calculated as a percentage of total $CD34^+$ cells.

TABLE 2. In Vitro Expansion of $CD34^+$ Cell Subsets in HUBEC Coculture versus Stroma-free and Non-brain Endothelial Cell Cocultures

	No. of Cells Procured x 10^5			
Culture Conditions	**Cell yield x 10^5**	**$CD34^+$**	**$CD34^+CD38^+$**	**$CD34^+CD38^-$**
Input	5.0	5.0	4.99 + 0.01	0.01 + 0.01
HUBEC Co-culture				
Day 7	72.3 ± 2.6	33.0 ± 1.0	28.9 ± 0.9	4.4 ± 0.2
Day 14	1163.0 ± 43.6	104.3 ± 57.0	87.3 ± 5.0	16.9 ± 1.0
Stroma-free				
Day 7	51.0 ± 1.3	15.1 ± 0.7	15.1 ± 0.6	0.02 ± 0.02
Day 14	396.0 ± 80.0	35.0 ± 2.8	35.0 ± 2.8	0
Non-CNS EC Co-culture				
Day 7	52.0 ± 2.8	18.1 ± 12.6	18.1 ± 12.6	0
Day 14	nd	nd	nd	nd

$CD34^+$ BM cells (5 x 10^5) were plated per culture treatment. Non-adherent cells were procured on day 7 and 14 of culture. Cells of each culture were stained for phenotypic analysis with FITC-conjugated CD34 (HPCA-2) plus PE-conjugated CD38). Stained cells were analyzed using two-color flow cytometry. The number of each immunophenotype was corrected to reflect the total number of cells procured/culture. Each point represents the mean number of positive cells from two different experiments. nd: no data

TABLE 3. Effects of HUBEC Co-culture on Hematopoietic Progenitor Cell Production in Comparison to Stroma-free and Human Non-brain Endothelial Cell Co-cultures.

Culture Conditions	Number of CFC x 10^4			
	CFU-GM	CFU-Mix	BFU-E	Total CFC
Input	3.7 + 0.9	0.5 + 0.2	0.6 + 0.3	4.7 + 1.4
HUBEC Co-culture				
Day 7	56.0 ± 2.2	2.6 ± 0.3	4.8 ± 0.6	63.4 ± 2.5
Day 14	2065.0 ± 49.0	64.5 ± 2.5	108.0 ± 9.5	2240.0 ± 70.7
Stroma-free				
Day 7	15.8 ± 4.5	0.5 ± 0.9	0.7 ± 0.7	17.1 ± 4.6
Day 14	200.0 ± 42.4	3.6 ± 0.2	19.8 ± 2.6	228.0 ± 41.0
Non-CNS EC Co-culture				
Day 7	22.2 ± 4.9	0.6 ± 0.3	4.8 ± 0.3	27.7 ± 6.2
Day 14	nd	nd	nd	nd

5×10^5 $CD34^+$ bone marrow cells were plated per culture treatment. Nonadherent cells were harvested on day 7 of culture. Nonadherent cells ($5\text{-}500 \times 10^2$) were cultured in 35-mm tissue culture dishes containing IMDM medium, 1% methylcellulose, 30% FCS, optimal concentrations of EPO, GM-CSF, IL-3 and SCF. The number of myeloid and erythroid colonies were counted after 14 days of culture, and based on the total number of viable cells per culture the number of colonies was corrected to reflect the total number of CFC per culture condition. Values represent the number of colonies of triplicate cultures from two different experiments.

nd: no data.

Cell Cycle Analysis

In another series of experiments, we studied the role of HUBEC co-culture on the cell cycle status of ex-vivo expanded $CD34^+$ cells. Analysis of $CD34^+CD38^-$ cells at day 0 demonstrated that 92.9% of the cells were in G_0, 5.9% were in G_1, and 1.2% were in G_2/S/M phase (Figure 5A). After 7 days of HUBEC co-culture, 55.2% of the $CD34^+CD38^-$ cells had entered G_1, 38.7% were in G_2/S/M phase, and only 5.8% remained in G_0 (Figure 5B). Similar analysis of the $CD34^+CD38^+$ subset indicated that 29.8%, 52.5%, and 17.2% of the $CD38^+$ cells were in G_0, G_1, and G_2/S/M phase at day 7, respectively. Analysis of $CD34^+$ cells from stroma-free and non-CNS endothelial cell cultures was not performed due to the relatively low/undetectable frequency of $CD34^+CD38^-$ cells following 7 days of culture.

Effect of ex vivo HUBEC Co-culture on the clonogenic capacity of $CD34^+CD38^-$ cells in methylcellulose CFC cultures

To determine whether 7 days of HUBEC co-culture could enhance the *in vitro* clonogenic capacity of $CD34^+CD38^-$ cells , we FACS sorted and collected $CD34^+$, $CD34^+CD38^-$, and $CD34^+CD38^+$ cell populations prior to and following 7 days of HUBEC co-culture. $CD34^+CD38^-$ and $CD34^+CD38^+$ cells could be easily collected in all samples analyzed. Sort windows were established to give a clear separation of $CD34^+CD38^-$ and $CD34^+CD38^{bright}$ cells, and therefore most of the $CD34^+CD38^{dim}$ cells were excluded from the analysis. Five hundred cells from each cell population were seeded into 1% methylcellulose containing Iscove's modified Dulbecco's medium (IMDM), supplemented with optimal concentrations of EPO, GM-CSF, IL-3, IL-6, and

SCF and scored for total CFC formation after 14 days of incubation. As shown in Table 4 and consistent with previous studies [29,30], very few, if any, steady state $CD34^+CD38^-$ cells (0.035%) were able to form colonies in standard methylcellulose based clonogenic media. Colonies derived from $CD34^+CD38^-$ cells were smaller on average than colonies derived from steady state $CD34^+CD38^+$ cells (cloning efficiency 11.9%) cultured under identical culture conditions. As expected, the $CD34^+CD38^+$ subset demonstrated a cloning efficiency which approximated the cloning efficiency of the entire steady state $CD34^+$ population (consisting of ~98 % $CD34^+CD38^+$ cells), thereby confirming that the large majority of colonies generated from steady state $CD34^+$ cells arise from the $CD34^+CD38^+$ subset with little or no contribution from the $CD34^+CD38^-$ subset.

In contrast to the results obtained culturing steady state $CD34^+CD38^-$ progenitor cells, when activated/expanded $CD34^+CD38^-$ cells were stringently re-selected from HUBEC monolayers after 7 day of coculture, a 685-fold expansion (from 0.035% cloning efficiency at day 0 to 24.0 % cloning efficiency at day 7) of CFC was detected. The number of assayable CFC was greater in cultures initiated with $CD34^+CD38^-$ cells (24.0% cloning efficiency) than in cultures initiated with $CD34^+CD38^{bright}$ cells (16.8%), but lower when compared to unsorted $CD34^+$ cells (40.1 %). This is most likely due to the fact that $CD34^+CD38^{dim}$ cells which have a high clonogenic potential comprise a significant portion of the day 7 $CD34^+$ cell pool and these were excluded from our analysis in the setting of stringent sort windows. In addition to increased colony numbers, an increase in colony size was also observed for cultures initiated with expanded and sorted $CD34^+CD38^-$ in comparison to $CD34^+CD38^+$ cells. Evaluation of the sorted $CD34^-$ cells from HUBEC co-cultures showed that this population was

practically devoid of CFC (0.3%). These plating efficiencies indicate that the majority of CFC generated following 7 days of HUBEC co-culture arises from the $CD34^{+}CD38^{-}$ and $CD34^{+}CD38^{dim}$ populations with significantly less contribution from the $CD34^{+}CD38^{bright}$ subset.

Table 4. Frequency of CFC in Sorted $CD34^+$ Cell Populations

Cell Population	CFC Frequency (%) Experiment 1	Experiment 2	Mean
	Input $CD34^+$ Cells (d-0)		
Unsorted $CD34^+$	13.4 ± 4.1	12.8 ± 3.7	13.1
$CD34^+CD38^-$	0.07 ± 0.01	0	0.035
$CD34^+CD38^+$	11.4 ± 3.5	12.4 ± 4.3	11.9
HUBEC Co-culture (d-7)			
Unsorted nonadherent cells	19.9 ± 1.1	19.9 ± 1.9	19.9
$CD34^+$	42.2 ± 2.6	37.9 ± 1.2	40.1
$CD34^+CD38^-$	28.9 ± 5.3	19.1 ± 2.8	24.0
$CD34^+CD38^+$	17.1 ± 4.6	16.4 ± 1.7	16.8
$CD34^-$	0.2 ± 0.2	0.4 ± 0.1	0.3

At day 0 and after 7 days of HUBEC co-culture $CD34^+$, $CD34^+CD38^+$, $CD34^+CD38^-$ and $CD34^-$ cell populations were collected by FACS sorting cells labeled with FITC-conjugated anti-human CD34 mAb and PE-conjugated anti-human CD38 mAb. Cells were cultured at 500 cells/dish in 1% methylcellulose containing Iscove's modified Dulbecco's medium (IMDM), supplemented with optimal concentrations of EPO, GM-CSF, IL-3, and SCF. The cultures were assessed at day 14 for colony-forming cells (CFC). Based on the total number of viable cells per culture the number of colonies was corrected to reflect the total number of CFC per culture condition. Values represent the number of colonies of triplicate cultures from two different experiments.

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Obviously, many variations and combinations of the invention can be seen from the above specific examples. The above examples are intended to disclose the best mode currently known to the inventors and is not intended to limit the invention.